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14. ABSTRACT

The aim of this study is to define whether exposing mice bearing breast tumors to simulated microgravity will increase nanoparticle drug convection from the blood to the tumor tissue by modifying the balance in the Starling-Landis equation. In this two-month period, breast tumors were established in mice using human (MDA-MB-231) and mouse (PY8119) cell lines and conditions for measuring interstitial fluid pressure (IFP) and blood and tissue distribution of fluorescent-labeled dextrans were set up. In preliminary experiments, no significant differences in tumor IFP and dextran uptake were observed between groups of mice submitted or not to microgravity simulation. Overgrown tumors (> 250mm3) often presented necrosis and were more prone to broader variations in IFP and dextran uptake, hence adjustments to the procedure are necessary. An improved technique for interstitial fluid withdrawal was developed, allowing larger and cleaner yields of fluid. Capillary hydrostatic pressure measurement is the next step. The results obtained create the basis for the performance of a solid study.

15. SUBJECT TERMS
breast cancer, simulated microgravity, interstitial fluid pressure, capillary hydrostatic pressure, nanodrug convection

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Period: Feb 15, 2009 – Feb 14, 2010

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Title: "Using Simulated Microgravity to Enhance the Effectiveness of Nanodrug Chemotherapy in Breast Cancer"

Table of Contents

INTRODUCTION

Cancer tissues usually present high interstitial fluid pressures (IFP) which reduce the transport of therapeutic agents by decreasing convection from blood into cancer tissues, increasing the possibility of poor treatment outcome in breast cancer [1]. The larger the molecular weight of the drug, the higher the detrimental effect of interstitial hypertension on drug delivery [2]. Several factors affect the convective transport of drugs across the vascular wall, which can be described by the Starling-Landis equation [3]. These factors include the IFP, the capillary hydrostatic pressure and the capillary and interstitial fluid (IF) colloid osmotic pressure, among others. Microgravity (or simulated gravity) exposure significantly increases the capillary osmotic pressure, which in turn compensate for increased IFP and improves the net transcapillary convection of drugs. We hypothesize that simulated gravity will improve the convection of nanoparticles in breast cancer, therefore improving drug delivery.

We will address this issue by submitting mice with implanted breast tumors of human or mouse origin to simulated microgravity and measuring a number of parameters in the Starling-Landis equation, including IFP, capillary hydrostactic pressure and the capillary and IF colloid osmotic pressure. In addition, we will measure under the same conditions the convective transport of nanoparticles by using dextrans of different molecular weights labeled with fluorochromes. The data obtained will provide evidence whether simulated microgravity improves drug convection to cancer tissues and can therefore be considered as a tool in the fight against cancer.

BODY

This project had its activities delayed as Dr Ugur Ozerdem left LJBI in mid 2009 leading to a process for change in PI that took several months. Dr Carvalho has taken the position of PI in this grant and the final approval for starting the activities was granted in December 23, 2009. Therefore, although this report covers the period of February 15 2009 to February 14 2010, the activities on this grant actually got started only recently, in January 2010.

In the revised statement of work, we had estimated the need for 3 months to undertake **Task 1** (Regulatory review and IACUC approval processes for mouse studies). However, because this task had already been accomplished during the process for approval of the change in PI, we were able to advance to the next tasks. We have concentrated our efforts in this period on establishing the simulated microgravity model (Task 2, Months 1-3) and establishing the breast cancer implantation in #1 mammary fat pads (Task 3, Months 3-10).

To accomplish these tasks, we hired Mr. Wisam Barkho, an animal technician with extensive previous experience in research with mouse models of cancer. In addition, we established consultation agreements with Dr Alan Hargens (UCSD), an expert in IFP measurements and IF sampling, and Dr Lesley Ellis (UCSD), an expert in mouse breast cancer cell lines.

Task 2: Establishment of the simulated microgravity model

Mr. Barkho was trained by Dr John Frangos and Diana Meays on the simulated microgravity model using hindlimb suspension. We have acquired the human breast cancer cell line MDA-MB-231 from

ATCC (Manassas, VA) and the mouse breast cancer cell line (PY8119) was provided by our consultant Dr Lesley Ellis, who also trained Mr. Barkho on the procedure to implant them in the mouse mammary fat pads (see **Task 3**). Mr. Barkho has succeeded in establishing the in vitro cultures of both the human (MDA-MB-231) and the mouse (PY8119) cell lines. We have therefore accomplished also **Task 2**.

Task 3: Establishment of the breast cancer in mouse #1 mammary fat pads

This task was supposed to be performed in Months 3-10. However, we took advantage of the rapid completion of Tasks 1 and 2 to move towards this goal already in January. We have succeeded in generating tumors with both cell lines (MDA-MB-231 and PY8119) injected in the mouse #1 mammary fat pads and have since then concentrated our efforts to set up the optimal conditions to perform the work as described. We have identified a number of variables and technical difficulties that need to be addressed before moving to the experiments themselves. We have also defined strategies that will enable us to decrease the number of animals in certain measurements, more specifically by simultaneously injecting dextrans of multiple sizes labeled with different fluorochromes in a single mouse, instead of having different animals for each dextran as originally conceived. We describe herein the work performed and results obtained during this process.

1. *Establishment of tumors in mice*

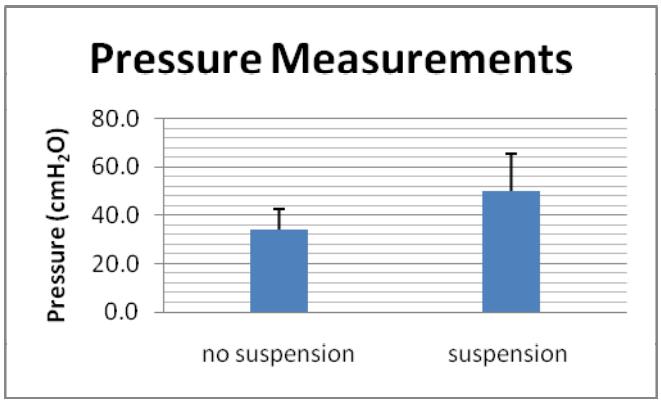
We have succeeded in growing both the MDA-MB-231 (human) and PY8119 (mouse) breast cancer cell lines in mice. The PY8119 cells grow fast, achieving sizes considered appropriate for measurements in 2 weeks or less. MDA-MB-231 cells take longer (4-6 weeks) to develop and achieve appropriate size, yet much faster than originally foreseen (80 days). We have observed that although the tumors keep growing after these periods, larger tumors start to develop necrosis in the core and therefore may not be appropriate for the study (see below). Tumors may also grow in different shapes (rounded, oval, elongated) and we observed that the volume of the tumor is a better indicative of suitability for measurements than its diameter. Based on the size requirements for proper measurements and the need to avoid necrosis, we have established that tumors not larger than 250mm^3 are the most suitable for the study.

2. *Measurement of Interstitial Fluid Pressure (IFP)*

Training in IFP measurements was provided by Dr Alan Hargens. We have then acquired a new system and catheters (Millar Instruments Inc., Houston, TX) and set it up with a BioPac MP150 acquisition device and interface (Biopac Systems Inc., Goleta, CA) for the measurements, as well as a water column device for calibration. We used this set up to conduct preliminary experiments to evaluate IFP in tumors of mice submitted or not to microgravity simulation (hindlimb suspension). In the first experiment, 5 mice bearing PY8119 tumors were suspended in microgravity simulation cages for 4 hours. Tumors were greater than 10mm in diameter and some tumors had external necrosis at the time of experiment. Five additional mice bearing PY8119 tumors were not suspended and used as controls. After this period, the tumor IFP was measured and results are shown in Figure 1. In both suspended and non-suspended mice, tumors presented a relatively high IFP. Although suspended mice showed a higher mean IFP, the difference between groups was not significant. Reliable measurements were obtained by inserting the catheter tip in three different locations in the tumor. There was a relatively wide range of IFP values in both groups and this may be due to variations in the size of the tumors and

to the fact that some of them presented necrosis. In addition, larger tumors apparently presented higher vascularization which may also interfere with the measurements. For this reason, as stated above, we will restrict the size and age of tumors in the future studies.

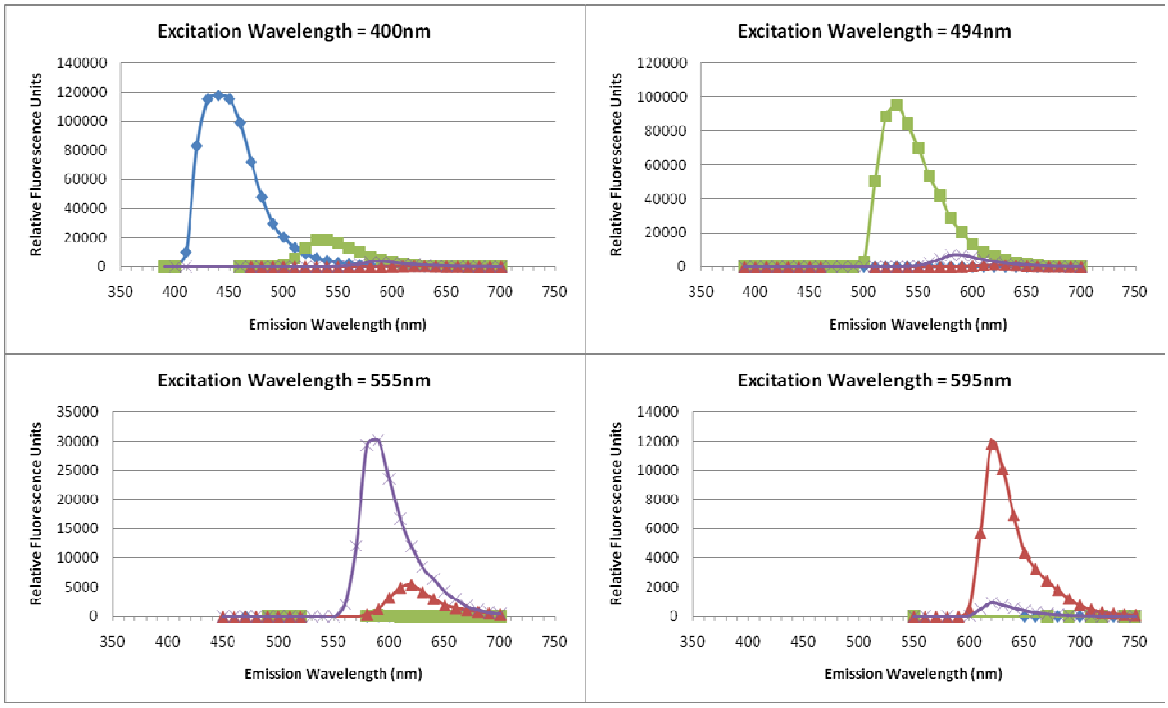
Figure 1. Interstitial Fluid Pressure of PY8119 tumors in mice submitted or not to hindlimb suspension.



3. *Establishing conditions for measuring the convection of fluorochrome-labeled dextrans to the tumor tissue*

In the original proposal, the convection of nanoparticles of different sizes to the tumor tissue would be evaluated by injecting different groups of mice with FITC-labeled dextrans. We realized that by using different fluorochromes for each dextran size we might be able to detect convection of each dextran in the same animal. With this strategy, we could substantially decrease the number of animals in the experiments without making changes to the procedure itself. To evaluate the suitability of this strategy, we selected dextrans of different sizes labeled with different fluorochormes (3KDa: cascade blue; 40KDa: FITC; 70KDa: Texas red; 2,000 KDa: TMR). First, we checked the sensitivity of detection of fluorescence emitted by each dextran-fluorochrome and found fluorescence signal can be detected even at concentrations of 10µg/mL or lower. We have then verified the potential interferences on each other by verifying the emission spectra of each dextran in a mixed solution containing the four dextrans, using pre-defined excitation wavelengths. The different emission spectra showed little overlap and therefore there seems to be little concern for interferences on each other (Figure 2). We have performed the same spectra checking using plasma from two mice injected with the mix of dextrans and the results were similar, except for the fact that the decay in fluorescence was different for each dextran, which is expected since they have different sizes and therefore different distribution properties, which is the very reason to use them to track the delivery of nanoparticles to tissues.

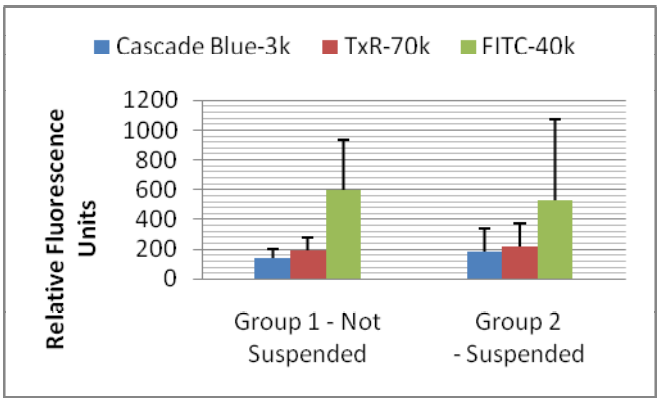
Figure 2. Emission spectra of mixed dextrans labeled with different fluorochromes.



Blue = Cascade Blue-3kDa; **Green** = FITC-40kDa; **Purple** = TMR-2000kDa; **Red** = TxR-70kDa. All dextrans used at 10mg/mL.

With these results in hands, we took advantage of the experiment described above (IFP measurement) to also perform a preliminary experiment to evaluate the delivery of dextrans to the tumor tissue. As described, 5 mice bearing PY8119 tumors were suspended in microgravity simulation cages for 4 hours. Tumors were greater than 10mm in diameter and some tumors had external necrosis at time of experiment. Five additional mice bearing PY8119 tumors were not suspended and used as controls. After this period, three fluorescent-labeled dextrans (3KDa-cascade blue, 40KDa-FITC and 70KDa-TxR) were injected at 0.5mg per mouse per dextran via tail vein and allowed to circulate for 15 minutes. After IFP was measured, tumors were harvested, weighed, homogenized in 2mL of saline and then centrifuged. 50µl of supernatant was read in a spectrophotometer (Spectramax, Molecular Devices Inc.). There was no significant difference in fluorescent dye uptake between the two groups (Figure 3). However, similar to the IFP measurements, there was variability in measurements among samples probably due to the same factors described above and therefore optimizations in the procedure are necessary.

Figure 3. Fluorescence detection of fluorochrome-labeled dextrans in tumor tissue (PY8119) homogenates in mice submitted or not to hindlimb suspension



A second experiment was performed on mice bearing PY8119 cells. Four mice were suspended in microgravity simulation cages for 4 hours and 4 mice were used as controls. Tumors had reached 10mm in diameter and showed no external necrosis. Blood was collected at two intervals (10 and 20 minutes) after fluorescent-labeled dextrans injection. Tumors were then harvested, weighed, homogenized in 2mL saline and centrifuged for supernatant. Figure 4 shows that the intensity of fluorescence in the plasma decays between 20-40% between 10 and 20 minutes after injection, indicating that the dextrans had been partially cleared from the circulation whether by tissue uptake or renal clearance. We were able to detect 3kDa and 40kDa, but not 70kDa or 2,000 kDa, dextran-derived fluorescence in the tumor tissue after 30 minutes of injection (Figure 5). This may indicate that these dextrans have not been efficiently delivered to the tissues due to their larger sizes. However, blood contamination may have also interfered with the measurements and optimization of the procedures is necessary to get definitive conclusions.

Figure 4. Decay of dextran-derived fluorescence intensity in the plasma as a function of sampling time

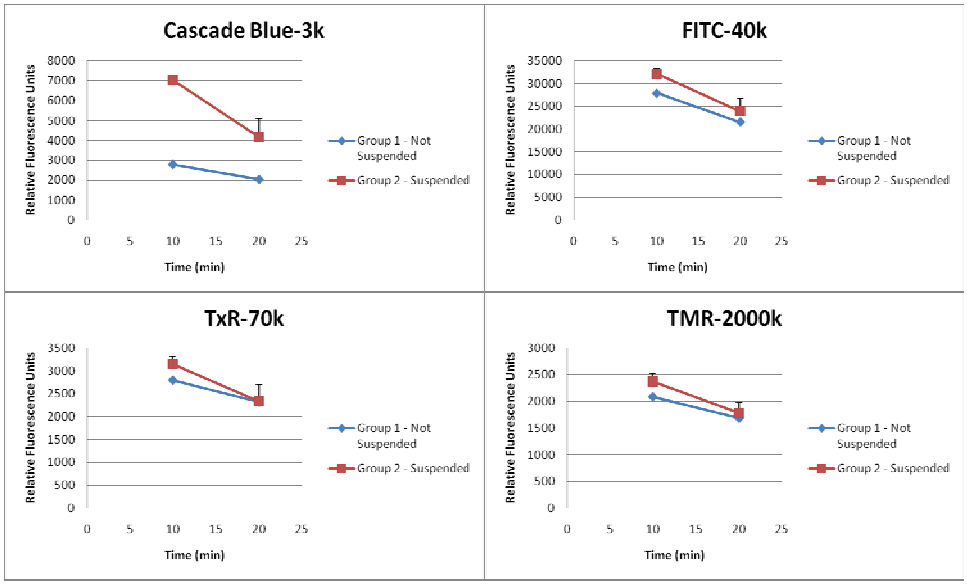
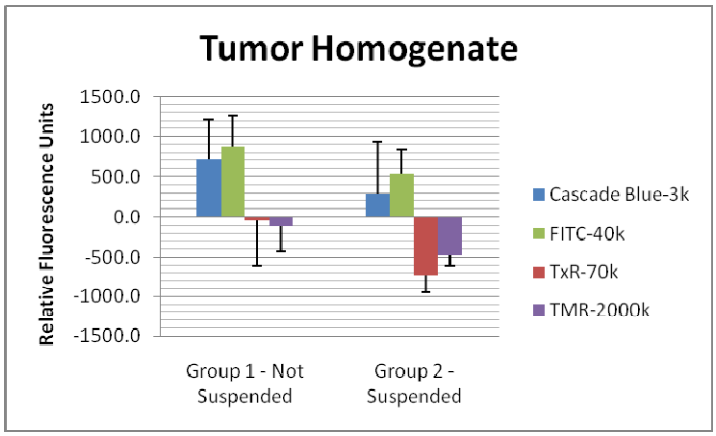
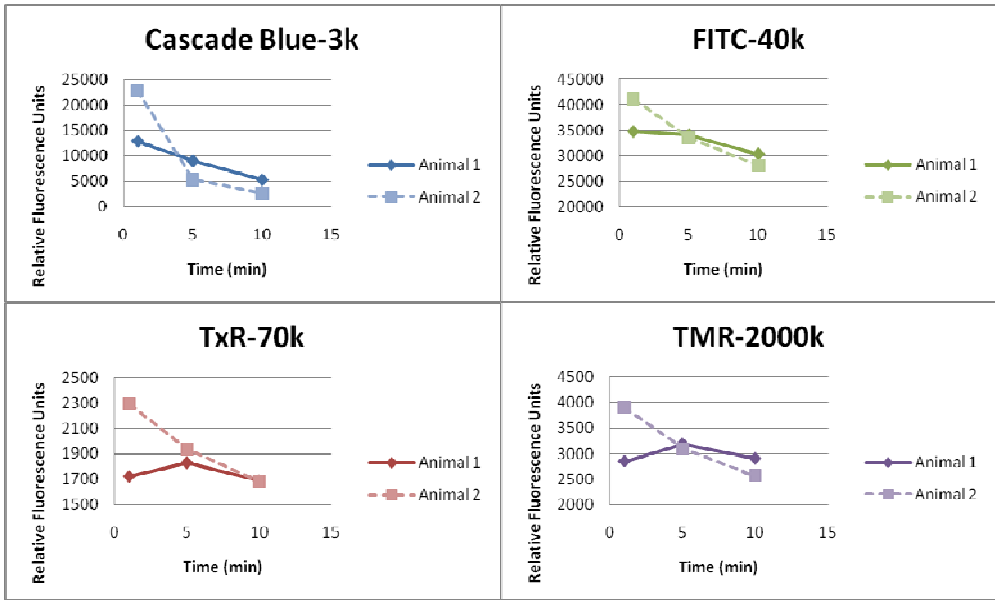


Figure 5. Dextran-derived fluorescence intensity in tumor tissue (PY8119) homogenates 30 minutes after intravenous injection



We have also used two mice to verify the decay of the dextran-derived fluorescences in periods shorter than 10 minutes and found out that they indeed decay very quickly (Figure 6), although the 1-minute measurements for animal 1 were not reliable. These data provide us with better background information to define proper timing for sampling. Because they show that relatively small delays in sampling generate substantial differences in fluorescence intensity, these data reinforce the need for a very precise time of sampling for all animals in the experiment.

Figure 6. Decay of dextran-derived fluorescence intensity in the plasma as a function of sampling time after intravenous injection

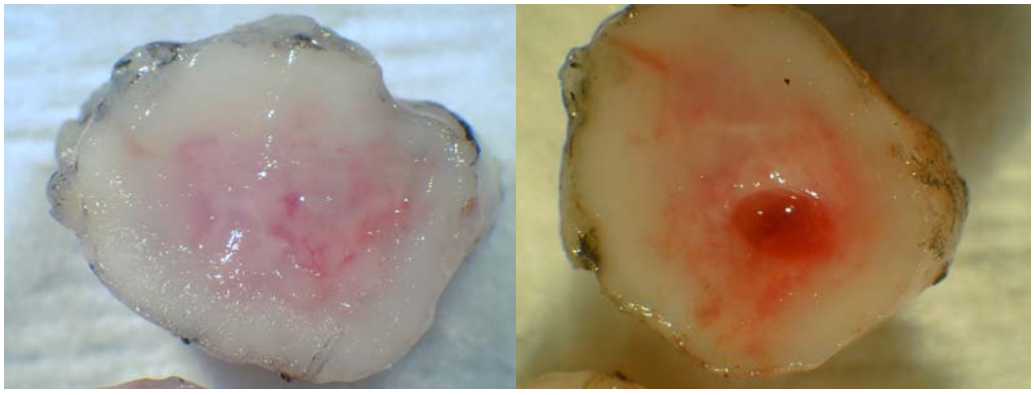


We have been dosing the fluorescent dextrans on tissue homogenates because it took us sometime to develop a proper technique to collect IF samples (see below). However, tissue homogenates may indeed represent a valuable alternative for measuring dextran convection as IF samples collected by the wick method provide only a limited amount of sample and there is a need for larger volumes to reliably measure fluorescence in the spectrophotometer.

4. *Establishing a reliable method do collect interstitial fluid sample in tumors*

Dr Alan Hargens also provided a demonstration of how to collect IF samples. However, the breast cancer tissue offers some hurdles for proper sampling, particularly the difficulty in getting samples without blood contamination. Mr. Barkho has attempted different procedures to get clean samples and has recently succeeded in getting substantial volumes with little blood contamination, basically by increasing the amount of blood taken by cardiac puncture for the fluorescent dextran measurements. The mouse is then terminated with pentobarbital and only then the wick is inserted in order to get the IF sample. The wick is inserted avoiding going through the core of the tumor, which we have observed is more vascularized and more susceptible to necrosis, whereas the outer rim has densely packed cells (Figure 7). In addition, by using extra wick material the amount of IF recovered can increase substantially. With these modifications of the original method we have succeeded in collecting up to 20µl of clean IF, up from the 3-5µl originally expected to be retrieved. A smaller amount of blood in the tumor also provides a cleaner sample and more consistent results for the dextran analysis. These modifications in the procedure actually do not change the way the mouse is handled, as cardiac sampling followed by euthanasia is the procedure described in the original protocol.

Figure 7. A relatively large (265mm^3) MDA-MB-231 tumor sliced to show the softer core which can become necrotic (ulceration seen in the right panel), whereas the outer rim has densely packed, fresh cells.



These IF samples have just been obtained and have not been used yet to measure the colloid osmotic pressure. This will be done soon in the laboratory of Dr Alan Hargens and the method will be then implemented at LJBI.

5. *Establishing the method for measuring capillary pressure*

This is the only method that has not been yet developed. We have been discussing the procedure with Dr Pedro Cabrales and we are set to start preliminary trials in the second half of March.

KEY RESEARCH ACCOMPLISHMENTS

- establishment and continued maintenance of in vitro cell cultures of PY8119 (mouse) and MDA-MB-231 (human) breast cancer cell lines
- establishment of tumors in mouse mammary fat pads using the PY8119 and MDA-MB-231 breast cancer cell lines, and delineation of the optimal conditions to perform measurements
- establishment of the method to measure IFP in breast tumors in the mouse
- establishment of an improved method to measure convection of fluorescent-labeled nanoparticles to the tumor tissue
- establishment of an improved method to collect IF samples

REPORTABLE OUTCOMES

Due to the issue of change in PI, this work has been ongoing for only a short period of time, therefore there is no reportable outcomes at this point.

CONCLUSION

Although the original proposal described the general methods to be used in this project, most of the procedures had not been previously standardized for use in breast tumors. Only with the clear and detailed definition of the experimental procedures it will be possible to achieve the proposed aims. Indeed, in case control measures are not taken, several factors may introduce variables and generate results either biased or difficult to interpret. The activities described in this report have been important to define the specific experimental conditions to be applied in our settings and to confirm that variables such as tumor size and age, small differences in the timing of sampling, blood contamination, necrosis, among others, are critical to control in order to achieve reliable and reproducible results. The results obtained so far create the basis for the performance of a solid study. In addition, some of the findings already provide contributions to the field, such as the description of an improved method for IF sampling in tumors.

It is noteworthy that a significant amount of data was generated in a short period of time. Indeed, within two months of the official start date (Dec 23, 2009), we have completed Tasks 1 and 2 (scheduled to be completed in 90 days) and have significantly advanced on Task 3.

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APPENDICES

None.